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PRINCIPAL INVESTIGATOR: Edward Thornborrow, MA
James Manfredi, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
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Edward Thornborrow, MA
James Manfredi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai School of Medicine
New York, New York 10029

E-MAIL:
et@doc.mssm.edu

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13. ABSTRACT ***(Maximum 200 Words)***

A significant percentage of breast tumors have been shown to be resistant to apoptotic stimuli. This resistance has been correlated with a decreased expression of the proapoptotic protein bax. A major regulator of *bax* expression is the tumor suppressor p53. When compared to alternate target genes data suggest that the *bax* gene is differentially regulated by p53. Our goal is to understand the mechanism by which p53 selectively regulates the transcription of the pro-apoptotic *bax* gene. We have identified the minimal p53 responsive element in the human *bax* promoter, and have found it to be unique in that it consists of three p53 half-sites instead of the typical two. In addition, this minimal element appears to bind with sequence specificity to at least two factors in addition to p53. One of these factors is the transcription factor Sp1 which appears to contribute significantly to the basal activity of the *bax* promoter. The other, Factor X (as yet unidentified) binds to sequences within the p53 response element that confer transcriptional repression in the absence of p53, suggesting that Factor X may posess potent oncogenic properties through its ability to effectively compete p53 for binding to the *bax* promoter.

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Differential Activation of p53 Target Genes in Breast Cancer

Introduction:

In the past decade, there have been great advances in the field of breast cancer. These advances largely have been in the fields of education and early detection. Unfortunately, however, the treatment of breast cancer continues to be problematic in that a significant proportion of tumors are resistant to chemotherapy. Evidence suggests that this resistance is due to a defect in the normal programmed cell death or apoptotic pathway. A complete understanding of the apoptotic mechanism, therefore, is essential for designing the most therapeutic regimen for the treatment of breast cancer. The protein bax is a key regulator of the apoptotic process, and low levels of this protein correlate with a decreased response to treatment and a decreased survival period in breast cancer patients. A major regulator of bax transcription is the tumor suppressor protein p53, and the work I am conducting in this fellowship addresses the transcriptional regulation of the *bax* gene by p53. As such, I hope to understand the mechanism by which bax levels are reduced in breast tumor cells, and then to use this information to elevate the level of activation of the *bax* gene, causing breast tumors to become sensitive to apoptotic stimuli like chemotherapy. I believe that completion of the work proposed in this fellowship will enhance our understanding of both the origins of breast cancer and how tumors respond to treatment. In addition, I believe this work will identify novel targets for therapeutic intervention in breast tumors that are otherwise resistant to current treatment.

Differential Activation of p53 Target Genes in Breast Cancer

Body:

A significant percentage of breast tumors have been shown to be resistant to apoptotic stimuli such as chemotherapeutic drugs. This resistance has been correlated with a decreased expression of the proapoptotic protein bax. Low bax levels have been associated with a decreased treatment response and a shorter survival time in women with metastatic breast adenocarcinoma. In addition, overexpression of bax in tumor cell lines sensitizes the cells to drug-induced apoptosis. A major regulator of *bax* expression is the tumor suppressor p53. p53 is a well-characterized transcription factor that can bind DNA in a sequence specific manner and activate the transcription of particular genes, including *bax*. When compared to alternate p53 targets such as the cyclin dependent kinase inhibitor *p21*, several lines of evidence suggest that the *bax* gene is differentially regulated by p53. Preliminary data presented in the fellowship application demonstrated that the breast carcinoma MCF-7 and MDA-MB-453 cell lines both exhibited a defect in the ability of wild-type p53 to activate transcription through both the intact *bax* promoter as well as the p53 response element in isolation from the promoter. This defect was specific for *bax* as p53 was capable of activating transcription of other p53-dependent target genes, such as the cyclin-dependent kinase inhibitor *p21*. The goal of the work being conducted in this fellowship is to understand the molecular mechanism by which wild-type p53 selectively fails to activate the transcription of the pro-apoptotic *bax* gene.

Work on this project began by identifying the minimal sequence within the *bax* promoter required to mediate p53-dependent transactivation. In contrast to other well characterized p53 response elements, which consist of two copies (half-sites) of sequences that closely resemble the palendromic decamer 5'-RRRCWWGYYY-3' (where R is a purine, Y is a pyrimidine, and W is an adenine or threonine) separated from each other by 0-13 bases, the minimal element from the *bax* promoter consists of three copies of this sequence, or three half-sites. Using this minimal sequence as a probe in electrophoretic mobility shift assays (EMSAs) with nuclear extract from MDA-MB-453 cells, a nuclear factor was identified that demonstrates marked sequence specificity for this p53 response element. This factor, referred to as BBF (Bax Binding Factor) in the original fellowship application, is specific for the p53-response element of *bax* as it fails to bind other well-characterized p53-response elements, including that of the cyclin-dependent kinase inhibitor *p21*. Five tasks were proposed in the original fellowship application to address the biological role of this binding factor in both breast tumor formation and resistance to treatment: 1) Identify additional wild-type p53 breast cancer cell lines that fail to activate transcription of the *bax* gene and fail to undergo p53-dependent apoptosis in response to DNA damage; 2) Explore a possible correlation between BBF levels and a cell line's ability to activate transcription of the *bax* gene and to undergo p53-dependent apoptosis; 3) Purify and clone BBF; 4) Determine the relevance of BBF to the apoptotic response in relevant breast cell model

systems; 5) Examine the potential correlation between BBF levels, bax levels, and p53 status in breast tumor biopsy samples as compared to normal mammary epithelium.

Mutational analysis of the p53 response element from the *bax* promoter has demonstrated that the BBF binding site is confined to the p53 half-site most proximal to the start site of transcription. This half-site consists of the sequence 5'-GGGCGTGGGC-3'. This sequence closely resembles the consensus DNA-binding sequence for the Sp1 transcription factor. EMSA analysis with antibodies directed against Sp1 demonstrated that in fact BBF is Sp1. With the fortuitous identification of BBF as Sp1, Task #3 has been successfully completed ahead of schedule. Subsequent to the identification of BBF as Sp1, we have found that Sp1 has a very high affinity for the p53 response element from the *bax* promoter, comparable to its affinity for its consensus DNA-binding sequence. Further, transfection assays in the Sp1 deficient Drosophila SL-2 cell line have demonstrated that Sp1 is capable of activating transcription through the p53 response element of the *bax* promoter in a sequence specific manner. Studies are currently underway in SL-2 cells to explore a potential transcriptional interaction between Sp1 and p53 on the *bax* promoter. These studies, however, may be complicated by interference from the recently identified drosophila homolog to the p53 protein. An additional approach, therefore, is being pursued which involves using an antisense-Sp1 expression vector to deplete Sp1 from various cell lines and subsequently assay the ability of p53 to activate *bax* expression and to trigger apoptosis (Task #4).

In addition to identifying BBF as Sp1, the mutational analysis of the p53 response element from the *bax* promoter allowed for the identification of an additional factor (Factor X) which appears to mediate sequence specific repression of transcription through the two distal p53 half-sites. In transient transfection assays, deletion of these half sites, leaving the Sp1 binding site intact, leads to an approximate 45-fold increase in basal levels of expression. In addition to identifying repressing sequences, these data also suggest that there is a significant activating role for the GC-rich sequence that binds Sp1. Insertion of this GC-rich sequence upstream of the adenovirus E1b-minimal promoter confers a 45-fold increase in basal expression levels when compared to comparable vectors not containing this sequence. Together, these data have caused us to propose a new model for the p53-dependent transcriptional activation of the *bax* gene. In the absence of p53, we propose that the *bax* promoter is repressed by Factor X, functioning through the two distal p53 half-sites. When p53 levels are elevated, as in response to DNA damage, it effectively competes with Factor X for binding to its response element, displacing it. In the absence of the repressor, p53 and Sp1 may cooperate to drive transcription of the *bax* gene, leading to apoptosis. If this model holds true, Factor X, with its ability to inhibit the p53-dependent activation of *bax*, may represent a protein with significant oncogenic properties. We, therefore, are vigorously attempting to address the biological significance of this factor in both breast tumor formation and resistance to treatment, using the same set of tasks originally

developed for BBF/Sp1. At the same time, we are continuing to pursue the significance of Sp1 in its own right.

In addition to the above research accomplishments, significant training accomplishments have been made. To be properly prepared for a successful career in the battle against breast cancer one should be familiar with both the history of research as well as the most current techniques used in the field. I believe that the work I am conducting is so intimately connected to both the origins and the treatment of breast cancer that it will provide me with the broad foundation of knowledge and technical skill that will allow me to continually identify important clinical aspects of breast cancer that can benefit from further scientific research. As an M.D./Ph.D. student at the Mount Sinai School of Medicine I am receiving specialized training that will allow me to traverse both the clinical and basic science worlds. The work I am conducting in the laboratory is an essential compliment to my clinical training that will continue to provide me with the skills to decrease the gap between benchtop discoveries and bedside cures. The experience of designing my research project and scrutinizing my experimental data is helping me to develop the analytical skills and experience necessary for a productive future battling breast cancer.

Differential Activation of p53 Target Genes in Breast Cancer

Appendices:

Key Research Accomplishments:

-Identified minimal p53 responsive element in the human *bax* promoter.

-Found the response element to be unique in that it consists of three p53 half-sites instead of the typical two.

-Demonstrated that Sp1 binds with sequence specificity to the same region of the *bax* promoter that is required for p53-dependent transcriptional activation.

-Found that in the absence of surrounding repressing sequences the Sp1-binding sequence contributes significantly to the basal activity of the bax promoter.

-Demonstrated that Sp1 can activate through this sequence in a sequence-specific manner.

-Identified sequences within the p53 response element of the bax promoter that confer transcriptional repression in the absence of p53.

Reportable Outcomes:

Edward C. Thornborrow and James J. Manfredi (2000), A high-affinity Sp1 binding site is located within the p53-response element of the human *bax* promoter (Abstract), *10th International p53 Workshop*, Monterey, California.

Cited Manuscripts and Abstracts:

Attached as pages 11-13

Poster Session: 2
Poster: 58
Edward C. Thornborrow

A high-affinity Sp1 binding site is located within the p53-response element of the human *bax* promoter.

Edward C. Thornborrow and James J. Manfredi. Derald H. Ruttenberg Cancer Center and Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY.

The tumor Supressor protein p53 has been shown to mediate transcriptional activation of many of its target genes via a response element containing two consensus half-sites, each half-site consisting of PuPuPuC(A/T)(A/T)GPyPyPy. In contrast, activation by p53 of the element in the human *bax* promoter requires a cooperative interaction between three adjacent half-sites (Thornborrow and Manfredi, 1999, J. Biol. Chem. 274, 33747). The half-site most proximal to the start site of transcription contains the sequence 5'-GGGCGTGGGC-3', which closely resembles the DNA-binding consensus sequence for the transcription factor Sp1. Electrophoretic mobility shift assays (EMSAs) using an oligonucleotide containing the p53-response element derived from the *bax* promoter as radiolabeled probe demonstrated that a factor from nuclear extracts bound to this probe and this DNA-protein complex is supershifted by an anti-Sp1 antibody. Competition experiments using oligonucleotides corresponding to a number of well characterized p53-response elements, including those from the *p21*, *cyclin G*, *IGF-BP3*, *mdm-2*, and *gadd45* genes, demonstrated that the binding of Sp1 to the p53 response element of the *bax* promoter was specific. Further, competition experiments with an oligonucleotide containing the Sp1 DNA-binding consensus sequence showed that the affinity of Sp1 for the p53 response element of *bax* is equal to if not greater than that of Sp1 for its consensus sequence. Deletion of the Sp1 binding site from the bax element results in a loss of demonstrable p53-dependent transcriptional activation in luciferase reporter assays. These results suggest that Sp1 and/or other Sp family members with similar DNA-binding characteristics, are important in regulating the p53-dependent transcriptional activation of the human *bax* gene.

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